



F9

## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :  C12N 15/55, 15/82, 9/16 C12N 5/10, 1/21, A01H 1/00		A1	(11) International Publication Number: WO 93/18158  (43) International Publication Date: 16 September 1993 (16.09.93)
(21) International Application Number: PCT/GB93/00432		(74) Agent: KEITH W. NASH & CO.; Pearl Assurance House, 90-92 Regent Street, Cambridge CB2 1DP (GB).	
(22) International Filing Date: 3 March 1993 (03.03.93)		(81) Designated States: AU, BG, BR, CA, CZ, FI, HU, JP, KR, NO, PL, RO, RU, SK, US, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).	
(30) Priority data: 9204583.0 3 March 1992 (03.03.92) GB		(71) Applicant (for AU CA GB IE only): UNILEVER PLC [GB/GB]; Unilever House, Blackfriars, London EC4P 4BQ (GB).  (71) Applicant (for all designated States except AU CA GB IE US): UNILEVER N.V. [NL/NL]; Weena 455, NL-3013 AL Rotterdam (NL).	
(72) Inventors; and (75) Inventors/Applicants (for US only) : HELLYER, Susan, Amanda [GB/GB]; "The Old Maltings", Upper Dean, Huntingdon, Cambridgeshire PE18 0NB (GB). SAF-FORD, Richard [GB/GB]; 10 Furness Close, Bedford, Bedfordshire MK41 8RN (GB). LOADER, Neil, Martin [GB/GB]; 20 Abbott's Way, Rushden, Northamptonshire NN10 9PL (GB). SLABAS, Antoni, Ryszard [GB/GB]; 1 Grove Court, Hett Village, County Durham DH6 5LE (GB).		Published With international search report.	

## (54) Title: RECOMBINANT PLANT ENZYME

## (57) Abstract

Disclosed is a nucleotide sequence encoding an enzyme having acyl-ACP thioesterase activity comprising nucleotides 169-1269 of the sequence shown in Figure 1 or functional equivalents thereof. Also disclosed is a polypeptide possessing said enzyme activity, vectors, host cells, and transgenic plants comprising the novel nucleotide sequence, together with a method of altering the characteristics of a plant and a method of producing the enzyme.

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo	KR	Republic of Korea	SE	Sweden
CH	Switzerland	KZ	Kazakhstan	SK	Slovak Republic
CI	Côte d'Ivoire	LJ	Liechtenstein	SN	Senegal
CM	Cameroon	LK	Sri Lanka	SU	Soviet Union
CS	Czechoslovakia	LU	Luxembourg	TD	Chad
CZ	Czech Republic	MC	Monaco	TG	Togo
DE	Germany	MG	Madagascar	UA	Ukraine
DK	Denmark	ML	Mali	US	United States of America
ES	Spain	MN	Mongolia	VN	Viet Nam
FI	Finland				

- 1 -

Title: Recombinant Plant Enzyme

Field of the Invention

This invention relates to the novel nucleotide sequence of a gene encoding a plant enzyme, functional equivalents thereof, vectors containing the novel nucleotide sequence, a method of producing the enzyme, a method of altering the characteristics of a plant and plants having been so altered.

Background of the Invention

Many plants and plant-derived materials (eg sunflowers, oil seed rape) are of commercial significance because they are a source of valuable vegetable oils. These oils (or lipids) consist of fatty acid residues (relatively long hydrocarbon chains) joined by ester links to propan -1, 2, 3 - triol (otherwise known as glycerol).

In some higher plants, de novo fatty acid biosynthesis is known to be catalyzed by two enzymes, acetyl-CoA carboxylase and fatty acid synthetase. The end product of de novo fatty acid biosynthesis is palmitoyl-ACP (Acyl Carrier Protein) which is then rapidly elongated to stearoyl-ACP. A highly specific stearoyl-ACP desaturase ensures that the only unsaturated fatty acid in high abundance is oleoyl-ACP. Another enzyme, acyl-ACP thioesterase is thought to play an important role in the chain termination of fatty acid biosynthesis by catalysing

- 2 -

the hydrolysis of acyl-ACP to free fatty acid and ACP. It is believed that subsequently the free fatty acid is incorporated into complex lipids.

Acyl-ACP thioesterases may be characterised by their preferred substrates. Thus acyl-ACP thioesterases showing preferential enzymatic activity for long chain fatty acids (eg C18 compounds) may be termed "long chain thioesterases".

Some long chain acyl-ACP thioesterases from plants have been identified and purified. These include the enzymes from Avocado and Safflower (Ohlrogge et al., [1978] Archives of Biochemistry and Biophysics 189, 382-391 and McKeon & Stumpf, [1982] Journal of Biological Chemistry 257, 12,141-12,147, respectively). However, no-one has been able to obtain the DNA sequence encoding such a long chain thioesterase. Such information would allow the application of modern recombinant DNA techniques to manipulate the enzyme characteristics (eg level of expression), and hence the characteristics of transgenic plants.

#### Summary of the invention

In one aspect the invention provides a nucleotide sequence, encoding an enzyme precursor having an acyl-ACP thioesterase activity, comprising the sequence of nucleotides 169-1269 shown in Figure 1 (Seq. ID No. 1) or functional equivalents thereof.

As will be apparent to those skilled in the art, functional equivalents of the nucleotide sequence of the invention include, for example: those nucleotide sequences

- 3 -

which encode the same polypeptide with the same activity (i.e. which cleave acyl-ACP molecules) but which, by virtue of the degeneracy of the genetic code, possess a different nucleotide sequence; sequences which encode substantially the same polypeptide but wherein there may be one or more conserved amino acid substitutions (i.e. the substitution of an amino acid for one with similar properties); sequences which encode substantially the same polypeptide (which preferably share at least 50% amino acid homology and more preferably at least 60% homology) but wherein there may be one or more minor deletions or truncations; and sequences which hybridize under standard conditions to the complement of nucleotides 169-1269. Typically such functional equivalents will have at least 75% nucleotide sequence homology and preferably at least 85% homology. An example of a functional equivalent is the sequence pNL3 (Seq. ID No. 3) shown in Figure 2.

A particular example of a functional equivalent is the sequence comprising the antisense equivalent to the sequence of the invention. Whilst antisense sequences are not generally understood to be functional equivalents, use of the term functional equivalent is intended for the purposes of the present application to encompass such sequences.

Another particular example of functional equivalents are those sequences which code for the mature enzyme rather than the enzyme precursor. Studies described below show that the precursor includes an N-terminal transit peptide of about 4kDa. Thus the N-terminal 27-37 amino acids (preferably the N terminal 29-35 amino acids) could be omitted. Alternatively, the transit peptide could be exchanged for the transit peptide of another protein (e.g.

- 4 -

that of ACP).

Preferably the sequence also comprises a suitable 5' untranslated region, including a promoter, to enable expression in appropriate host cells.

Preferably the sequence also comprises a suitable 3' untranslated region. As well as a stop codon, this 3' untranslated region can comprise other signals, such as a polyadenylation signal.

In another aspect, the invention provides an enzyme precursor having an acyl-ACP thioesterase activity, comprising the amino acid sequence shown in Figure 1 (Seq. ID No. 1) or functional equivalents thereof.

As described previously, functional equivalents can include mature enzymes lacking the N terminal transit peptide (which is thought to be cleaved between Ala33 and Val 34, or in the vicinity thereof). Other functional equivalents include chimaeric polypeptides comprising the transit peptides of other proteins (such as that from ACP).

An example of such an enzyme precursor is the acyl-ACP thioesterase from Brassica napus (oil seed rape), the gene for which has been cloned and sequenced by the inventors. The sequences of the full length cDNA clones obtained, pNL2 and pNL3, are shown in Figures 1 and 2. A number of sequence variations can be seen (detailed in Figure 3). Some of these nucleotide sequence differences result in different deduced amino acid sequences, as shown in Figure 4.

- 5 -

It is thought that all these sequence variations are manifestations of the same phenomenon: it is believed that the B. napus acyl-ACP thioesterase enzyme is encoded by a multi-gene family (i.e. there are a number of acyl-ACP thioesterase allelic genes in B. napus). This has already been demonstrated for the B. napus ACP gene (Safford et al., (1988), European Journal of Biochemistry 174, 287-295). There is also evidence to suggest that there are several (10-20) copies of the thioesterase gene in the B. napus genome (see Figure 6).

Thus in a specific embodiment the invention provides a nucleotide sequence encoding an enzyme precursor having acyl-ACP thioesterase activity and comprising the amino acid sequence shown in Figure 1, or functional equivalents thereof.

In another aspect, the invention provides a vector containing a nucleotide sequence in accordance with the invention.

A further aspect of the invention comprises a cell transformed with the vector defined above and thus capable of expressing an enzyme precursor with acyl-ACP thioesterase activity.

Such a transformed cell may be of bacterial, fungal, plant or animal origin.

Such transformed cells can be grown in cultures using methods well known to those skilled in the art. Thus, in the case of a transformed plant cell, one may obtain a transgenic plant.

- 6 -

In yet another aspect, the invention provides a method of producing an enzyme precursor having acyl-ACP thioesterase activity comprising the steps of: inserting a sequence encoding the enzyme precursor or a functional equivalent into a suitable expression vector; transforming a suitable host cell with said vector; growing said transformed host cell in suitable culture conditions; and obtaining the enzyme precursor from the host cells and/or from the culture medium.

Preferably, a sequence is employed which encodes a functional equivalent so as to produce the mature enzyme.

In view of the disclosures made herein, suitable vectors, host cells and culture conditions will be apparent to those skilled in the art.

It may be advantageous to obtain the enzyme precursor free from contamination by other plant proteins or products, thus in a preferred embodiment the enzyme precursor is expressed in an animal or bacterial cell. Most preferably a microorganism is used to express the enzyme precursor. Conveniently the precursor is secreted.

The invention has a number of applications. It might prove possible, by under- or over-expressing this enzyme precursor (or mature enzyme), to alter the properties of the storage lipid molecules of the plant in which the enzyme precursor is expressed. For instance, by over-expressing the enzyme precursor it might be possible to bring about premature termination of the hydrocarbon chain elongation during fatty acid biosynthesis, thus resulting in lipids with shorter hydrocarbon chain fatty acid components. Such "medium chain" lipids are useful as

- 7 -

"hardening stock" in the production of margarines and detergents. Alternatively, by use of an anti-sense construct, it might be possible to reduce the levels of active enzyme in the plant, resulting in the increased production of longer chain fatty acids. Equally, because the enzyme catalyses the last stage in fatty acid biosynthesis, it should prove possible to modify the fatty acid levels of the plant.

Typically the plants to be altered could include rape, sunflower, safflower, soybean, peanut, cotton, oil palm or corn.

Thus in another aspect the invention provides a method of altering the characteristics of a plant, comprising introducing into the plant the sequence of the invention or a functional equivalent thereof, so as to alter the level of acyl-ACP thioesterase activity.

Preferably the enzyme precursor or function equivalent is selectively expressed in the plant seeds. This can be achieved by expressing the precursor or functional equivalent under the control of a seed specific promoter. Several such promoters are known (e.g. those for the ACP, napin and cruciferin genes).

The characteristics one might expect to alter, in addition to acyl-ACP thioesterase activity are those influenced by levels of the enzyme precursor or mature enzyme, such as fatty acid yield and composition.

Preferably the characteristic altered is the storage oil yield or composition of the seeds of the plant.

- 8 -

The acyl-ACP thioesterase from B. napus exhibits a strong substrate specificity, with oleoyl-ACP (18:1-ACP) being the preferred substrate. Ideally, the product hydrocarbon should have chain lengths shorter than 18 carbon atoms. One plant which contains storage lipids with fatty acid components of the preferred length is Cuphea. Thus, by use of DNA probes based on knowledge of the B. napus enzyme sequence, and by use of antibodies raised against the purified B. napus enzyme, it might be possible to identify clones containing the sequence encoding the corresponding Cuphea enzyme, which could be used to modify fatty acid biosynthesis in the desired manner.

The invention is further described by reference to the following examples and drawings of which:

Figure 1 shows the DNA sequence of cDNA clone pNL2 which encodes the B. napus acyl-ACP thioesterase gene and the deduced amino acid sequence of the enzyme,

Figure 2 shows the nucleotide sequence of cDNA clone pNL3 which encodes an allelic variant of B. napus-acyl-ACP thioesterase, and the deduced amino acid sequence,

Figure 3 shows the variations in nucleotide sequence between pNL2 and pNL3,

Figure 4 shows the variations in the deduced amino acid sequence of the proteins encoded by pNL2 and pNL3

Figure 5 shows the amino acid sequences obtained by direct sequencing of peptides generated by enzymatic digestion of B. napus acyl-ACP thioesterase, with boxes indicating residues which show variation from the amino acid sequence

- 9 -

expected from the cDNA sequences, and

Figure 6 shows a B. napus genomic Southern blot probed with an oilseed rape thioesterase probe.

Example 1

Figures 1 and 2 show the nucleotide sequence of pNL2 and pNL3 respectively, full length cDNA clones of the B. napus acyl-ACP thioesterase gene, together with the deduced amino acid sequences of the respective proteins encoded. Several differences are observed between the amino acid sequence obtained directly from the protein and that deduced from the cDNA clones (see Figure 5).

Clones pNL2 and pNL3 were obtained in the following manner.

Poly A<sup>+</sup> RNA was isolated from 25-30 days after flowering (D.A.F.) B. napus embryos, as previously described [Hall et al. (1978), Proceedings of the National Academy of Sciences 75, 3196-3200], and used to construct a lambda gt10 cDNA. The cDNA was made essentially according to the manufacturer's instructions (Pharmacia) and then ligated into the vector and packaged in vitro. Basically, double stranded oligo dT-primed cDNA was synthesised from poly A<sup>+</sup> RNA according to Gubler and Hoffman [Gene (1983) 25:203-269]; EcoRI linkers were ligated to the ends, and the resulting material cloned into a bacteriophage vector, lambda gt10 (Amersham International).

Approximately 50,000 clones were screened using an oligonucleotide probe (RAAT 3AS - see below, Seq. ID No. 5), derived from the rape thioesterase peptide VMMNQDT.

- 10 -

Several positive clones were obtained and the longest (1200bp) was subcloned into pTZ18R (U.S.B.) making a construct termed pNL1. Following sub-cloning into M13, overlapping fragments of pNL1 were sequenced in both directions.

A  
A T A C  
RAAT 3AS 5' GTGCCCTGGTTCAATCATGAC 3' 21 mer  
T

Translation of the DNA sequence gave rise to an open reading frame (ORF) of 233 amino acids plus a 325bp 3' non-coding sequence. The ORF contained a number of sequences that had been identified by direct amino acid sequencing of rape acyl-ACP thioesterase peptides. pNL1 was therefore determined to code for rape acyl-ACP thioesterase.

A second B. napus cDNA embryo library was constructed in lambda ZAPII (Stratagene), according to the manufacturer's protocol. 100,000 plaques were screened with a 730bp BglII pNL1 fragment and eight positive clones were obtained. The eight clones were purified to homogeneity and in vivo excised, according to the Stratagene protocol, to give rescued plasmids containing the cDNA insert within the polylinker. Restriction analysis revealed that two plasmids, pNL2 and pNL3, contained inserts of 1560bp and 1460bp respectively. pNL2 and pNL3 were subcloned into M13 and sequenced. Translation of the resulting DNA sequences showed both pNL2 and pNL3 to contain ORFs of 363 amino acids, but differing in the length of their 3' non-coding sequences.

- 11 -

Neither pNL2 nor pNL3 contained an initiating methionine and, in order to obtain this, the technique of 5' RACE (Rapid amplification of cDNA ends) was employed, essentially as described by Frohman *et al.* [Proceedings of the National Academy of Sciences 75, 8998-9002]. 1ug of 25-30 D.A.F. B. napus embryo poly A<sup>+</sup> RNA was reverse transcribed at 37°C for 60 min with 200 units M-MuLV Superscript reverse transcriptase (BRL) and 500 pmoles random hexamers as primer. Excess primers were removed on a Centricon 100 column and cDNA precipitated with isopropanol. After resuspension in 10ul TE buffer, the cDNA was tailed using 10 units terminal transferase (BRL) in buffer (BRL) and 200uM dATP at 37°C for 10 min. After treatment at 65°C for 5 min the mixture was diluted to 500 ul with TE. 10ul mixture was used in a 100ul second strand Polymerase Chain Reaction (PCR) with 50 pmol rape thioesterase specific primer PTB5

[5' GATTAGCAATTGTCTCGACGG, Seq. ID No. 6],  
50-pmol R primer

[5' AAGGATCCGTCGACATC Seq. ID No. 7]

and 5pmol T<sub>17-R<sub>0</sub>-R<sub>I</sub></sub> primer

[5' AAGGATCCGTCGACATCGATAATACGACT

CACTATAGGGATTTTTTTTTTTTTTTT, Seq. ID No. 8].

The mixture was heated to 95°C for 7 min then held at 72°C. 10ul 10 x PCR buffer (BRL), 200uM dNTPs and 2.5 units Taq polymerase were added, heated at 45°C for 2 min then 72°C for 40 min. To PCR amplify the products, the mixture was heated to 94°C for 45 min to denature cDNA, primer annealed at 50° for 25 min and extended at 72°C for 3 min. This cycle was repeated 34 times, followed by final extension at 72°C for 10 mins.

1ul of the above PCR products was used in a second amplification containing 200uM dNTPs, 2.5 units Taq, 50

- 12 -

pmole R<sub>1</sub>  
[5' GACATCGATAATACGAC, Seq. ID No. 9],  
50 pmole rape thioesterase specific primer PTB2  
[5' CAAGAATTAACAGGGCTGATG, Seq. ID No. 10],  
(internal to PTB5), PCR buffer added to 50ul. Mixture  
heated 94° for 45 min, 50°C for 30 min and 72°C for 1 min  
- 28 cycles followed by final extension at 72° for 10 min.  
The products of this second PCR amplification, after size  
selection on agarose gels, were ligated into pT7 Blue (AMS  
Biotechnology). Recombinants were identified by PCR using  
50 pmol R<sub>1</sub> primer and 50 pmol PTB3, another rape  
thioesterase specific primer [5' GCGATTAACCAGGGATGAAAAG,  
Seq. ID No. 11]  
internal to PTB2. This mixture was heated to 94°C for 1  
min, 55°C for 1 min, 72°C 1.1/2 min. This cycle was  
repeated 24 times and the products subjected to a final  
extension at 72° for 10 min.

144 colonies were screened and 15, containing the largest  
inserts, were selected for DNA sequence analysis. The 2  
longest inserts, RACE 6 and RACE 1, were both found to be  
180bp longer than the 2 cDNA clones pNL2 and pNL3 at the  
5' end and to contain a putative initiating methionine. A  
160bp overlapping region between the cDNAs and the RACE  
products enabled the origin of the 2 RACE products to be  
determined. Thus RACE 6 was identical in sequence to pNL2  
and RACE 1 was identical to pNL3.

The complete nucleotide and deduced amino acid sequences  
of pNL2 and pNL3 are shown in Figure 1 and Figure 2  
respectively. The 2 composite clones pNL2 and pNL3  
represent entire copies of the structural genes of rape  
acyl-ACP thioesterase. Excluding poly(A) tails, pNL2  
contains 1642bp and pNL3 1523bp. Both pNL2 and pNL3 have

- 13 -

putative initiation methionines at nucleotide 169, which show good homology to the plant consensus translation initiation sequence, and to encode open reading frames (ORFs) of 366 amino acids. The two clones differ in the length of their 3' non-coding sequences. The open reading frames of pNL2 and pNL3 encode polypeptides of 41,960 Da and 41,983 Da respectively and these represent the precursor form of the thioesterase. On SDS-PAGE, mature rape thioesterase migrates with a molecular weight of 38 kDa (see below) suggesting a transit peptide of about 4 kDa. Since the N-terminus of the mature protein is blocked to Edman degradation the precise cleavage site of the precursor polypeptide could not be determined.

Figure 3 shows the nucleotide sequence variation between cDNA clones pNL2 and pNL3. Some of these sequence differences are observed to be in the ORFs, and result in different deduced amino acid sequences for the polypeptides encoded by pNL2 and pNL3. These differences are illustrated in Figure 4.

To further characterise the protein, it was highly purified, as described below. (All steps were carried out at 4°C unless otherwise stated.)

#### Step 1: Preparation of cell free extract

Rape seed (100g), which had been harvested between 40-60 days post anthesis (after flowering), and stored at -70°C, was homogenised in 250ml 20mM potassium phosphate, pH 7.3, containing 2mM dithiothreitol and 0.2mM phenylmethylsulfonyl fluoride using a polytron at maximum speed. The resulting homogenate was stirred for 30 minutes with Triton X-100 (stock solution 20% v/v) at a

- 14 -

final concentration of 1% v/v. After centrifugation at 20,000g for 30 minutes the supernatant was filtered through glass wool to remove the floating lipid layer.

Step 2: Ammonium Sulphate fractionation

The filtrate was brought to 30% saturation by the addition of solid ammonium sulphate and the suspension was stirred for 30 minutes prior to centrifugation at 20,000g for 30 minutes. The supernatant was refiltered through glass wool and brought to 50% saturation with solid ammonium sulphate. After stirring for 45 minutes the extract was centrifuged for 20 minutes at 30,000g. The pellets were stored at -20°C until required.

Ammonium sulphate pellets were resuspended in Buffer A (20mM bis-Tris HCl, pH 6.5, containing 2mM dithiothreitol and 0.2mM PMSF) and centrifuged at 30 000g for 10 minutes to remove particulate matter.

Step 3: Fast Flow Q-Sepharose chromatography

The supernatant was diluted with Buffer A until the conductivity was less than  $20 \times 10^{-4}$  ohms (final volume 200ml) and loaded onto a Fast Flow Q-Sepharose column (2.5cm x 12cm) equilibrated in 20mM bis-Tris HCl, pH 6.5. After loading, the column was washed with equilibration buffer until absorbance at 280nm was less than 0.15. Enzyme activity was eluted with a 300ml gradient from 0-0.5M NaCl in Buffer A. Fractions (5ml) were collected and assayed for activity. The flow rate throughout was 1.3ml minute<sup>-1</sup>. The unbound sample was assayed for activity and typically less than 10% of the activity loaded did not bind. Thioesterase activity eluted between 150 and 300mM

- 15 -

NaCl. Fractions containing activity were pooled (115-120ml) and stored overnight at -70°C.

Step 4: ACP-Sepharose chromatography

The Q-Sepharose pooled fractions were rapidly thawed and applied to an ACP-Sepharose column (1cm x 13cm) equilibrated in 20mM potassium phosphate pH 6.5. After loading, the column was washed with 0.1M potassium phosphate, pH 6.5, alone. Activity was eluted with 0.3M potassium phosphate pH 8.0. All buffers contained 2mM DTT and 0.2mM PMSF and the flow rate throughout was 0.5ml minute<sup>-1</sup>. Fractions (5ml) were collected and assayed for activity. Using freshly synthesised affinity matrix, greater than 95% of the activity loaded was bound and no activity was detected in the wash fractions. Typically, 30-40% of the activity loaded was eluted. Fractions containing activity were pooled (25-30ml) and stored at -20°C overnight. Typically 70% of activity was retained after thawing.

Step 5: F.P.L.C Mono P chromatography

The ACP-Sepharose pool was dialysed against 2.5l 25mM bis-Tris, iminodiacetic (pH 7.1) acid for 4.5 hours with one change of buffer after two hours. The conductivity of the dialysed sample was checked and, if necessary, the sample was diluted with 25mM bis Tris pH 7.1 until the conductivity was less than  $10 \times 10^{-4}$  ohms. The sample was loaded via a 50ml superloop onto a Mono P column (HR 5/20) equilibrated in 25mM bis-Tris iminodiacetic acid pH 7.1 at a flow rate of 1 ml minute<sup>-1</sup> at room temperature. The column was washed with equilibration buffer and activity was eluted with 50ml 10% polybuffer 74 (v/v)/iminodiacetic

- 16 -

acid, pH 4.0, at a flow rate of 1 ml minute<sup>-1</sup>. Fractions (1ml) were collected into tubes which contained 50ul 1M bis-Tris pH 9.9 to immediately neutralise the elutant since thioesterase activity appeared to be unstable at low pH. To determine the pH elution gradient, the column was calibrated with a blank run prior to loading the enzyme. Under these chromatographic conditions, all activity bound to the column and typically 40-50% of the activity loaded was recovered in five fractions.

Active fractions eluted from the Mono P column were immediately concentrated on an Amicon 10 microconcentrator, pre-coated with protein to prevent non-specific binding. Concentrated sample (final volume 1ml) was brought to 0.2M NaCl and stored at -20°C overnight. Samples stored at -20°C lost almost no activity while those stored at 4°C lost typically 60% activity.

#### Step 6: F.P.L.C. Superose 12 gel filtration

On thawing, the Mono P concentrated pool was centrifuged on an MSE centaur for 5 minutes prior to loading onto two Superose 12 gel filtration columns HR10/30 (1 x 30cm) in series. To obtain maximum purification, care was taken not to overload the column and therefore 200ul aliquots of the supernatant were loaded for each run. The column running buffer was 50mM sodium phosphate, pH 7.2, containing 150mM NaCl. Fractions (200ul) were collected and assayed for activity. Activity eluted as one peak over 5-6 fractions and typically 45% of the activity loaded was recovered. Five runs were necessary to purify the concentrated sample.

Once partially purified, the enzyme was further

- 17 -

characterised. The intact enzyme was found to have a molecular weight of about 70 kDa, being composed of a dimer of approximately 38kDa sub-units. A degraded form of the enzyme (33kDa) was found to co-purify with the intact 38kDa subunits. The pH optimum was 9.5. As with other plant acyl-ACP thioesterases so far described, the preferred substrate of the B. napus enzyme was found to be oleoyl-ACP (18:1-ACP).

The partial amino acid sequence of the purified protein was determined as described below.

The highly purified preparation, after Mono P chromatography, was concentrated and reductively alkylated with 4-vinyl-pyridine. Protein samples were loaded directly, via a 100ul loop, onto a Brownlee C-8 (C03-032) column pre-equilibrated in 0.1% TFA (v/v). Flow rate was 0.1ml min<sup>-1</sup>. The column was washed with 0.1% TFA until absorbance at 214nm was close to baseline. Protein was eluted with a gradient of increasing Buffer B (90% acetonitrile; 0.085% TFA) 0-70% over 35 min. It was necessary to pause the gradient for 40-45 min at 38% Buffer B to elute isocratically a major absorbance peak which contained no protein and was probably a contaminant from 4-vinyl-pyridine. Protein peaks were collected manually into Eppendorf tubes, fractions containing acyl-ACP thioesterase polypeptides (38 and 33kDa) were pooled.

After reversed-phase chromatography, the pooled purified enzyme preparation was brought to near dryness on a Univap concentrator. The appropriate digestion buffer, detailed below, was added and the pH was measured using indicator paper and adjusted with 1M NaOH. During the concentration step, a heavy precipitate formed which disappeared on

- 18 -

neutralisation. The final volume of samples prepared for digestion was 100-150ul.

Preparations containing 3-7ug (80-180 pmoles) of purified thioesterase polypeptides (38 and 33kDa) were digested with 0.15ug Endoproteinase Lys-C (Boehringer Mannheim). To check for auto-digestion a control sample was prepared with 0.15ug protease in 150ul 100mM Tris/HCl (pH 8.5), 2mM EDTA (digestion buffer). Incubations were left at 37°C for 10-16 hours. Additional Endoproteinase Lys-C (0.15ug) was added to the thioesterase and control incubations. Digestion was allowed to proceed at 37°C for 40 hours. Samples were stored at -20°C prior to reversed-phase chromatography.

Similarly a preparation, containing 9ug (240 pmoles) of purified enzyme (38 and 33kDa polypeptides), was digested with 0.18ug TPCK-trypsin (Cooper Biomedical) overnight at 37°C. A control sample was prepared with 0.18ug TPCK-trypsin in 100ul 0.2M ammonium carbonate/1mM calcium chloride (digestion buffer) and incubated as for the protein sample. Fresh TPCK-trypsin was prepared and a further 0.18ug was added to the protein and control incubations. Both samples were left at 37°C for 8 hours. Samples were stored at -20°C prior to reversed-phase chromatography.

Reversed-phase chromatography was performed at room temperature on Gilson HPLC equipment adapted for microbore column chromatography. Samples were loaded, via a 100ul loop, onto an Aquapore RP 300-C8 microbore column (250 x 1mm id; 7μ) pre-equilibrated in 0.1% TFA. Flow rate was 0.1ml/minute. Peptides were eluted with a gradient of increasing buffer B (90% acetonitrile; 0.085% TFA) 0-70%

- 19 -

over 100 minutes. Absorbance was monitored at 214nm and peaks were collected manually into Eppendorf tubes. Prior to loading sample, acetonitrile gradients were performed until a reproducible low baseline was obtained.

N-terminal Protein Sequencing was performed on an Applied Biosystems model 475 protein sequencer as described previously (Cottingham et al., [1988] Biochim. Biophys. Acta 954, 201-207). A number of peptide sequences were obtained. These are shown in Figure 5 and are Seq. ID Nos 12 (peptide 26), 13 (peptide 15), 14 (peptide 30), 15 (peptide 34) and 16 (peptide 39). The residues boxed in the tryptic peptides are different to those expected from translation of the cDNA sequences. These differences are thought to be due to the allelic nature of the gene.

From the partially determined amino acid sequence, it was possible to identify nucleic acid sequences encoding the enzyme. The techniques and methods of accomplishing this are well known to those skilled in the art. Figure 5 shows a genomic Southern blot probed with thioesterase - specific probe. Molecular standard markers (obtained by a PstI digest of lambda DNA) are shown on the right hand side with their size shown in kilobases. The middle four lanes represent controls having a known copy number (1, 2, 5 or 10) whilst Hpa, Sac and Bam are restriction digests of rape genomic DNA resulting in 12, 17 and 16 copies respectively per haploid genome. The probe used was a 764bp fragment generated by PCR, using primers PTB1 AS and PTB2AS (Seq. ID Nos 17 and 18 respectively), shown below:

PTB1AS 5' CCGAGCTCGTCGATTGATGGAAGA 3' 24-mer 201-224bp

PTB2AS 5' TTGGTACCGGTAAGCTTGAGATC 3' 24-mer 942-965bp

- 20 -

Example 2

This example describes the construction of a plant transformation vector containing an antisense rape thioesterase fragment linked to a seed-specific acyl carrier protein (ACP) promoter for use in altering fatty acid composition and oil yield in transgenic oil seeds.

The 764 bp fragment of pNL2 (described above) was produced by PCR amplification using the primers PTB AS1. PTB AS1 and PTB AS2 incorporated SacI and Kpn1 restriction sites respectively into the fragment. A 100ul PCR reaction contained 10ul 10x reaction buffer (Stratagene), 200uM dNTPs, 50 pmole PTB AS1, 50 pmole PTB AS2, 100pg pNL2, 2.5 units Taq (Stratagene). Reaction conditions were 92° for 45 sec, 55°C for 45 sec, 72°C for 90 sec for 25 cycles followed by 72°C for 10 min. The 764 bp fragment was recovered via DEAE paper from agarose gels, digested with SacI and Kpn I, re-run on agarose gels and recovered from DEAE paper.

A 1.4kb BamHI-BglII fragment of pTZ5BS [de Silva et al., (1992) Plant Molecular Biol. 18:1163-1172], representing a seed specific ACP promoter sequence, was ligated into BamHI-restricted pTZ18R [Mead et al., (1986) Prot. Engin. 1: 64-74] to produce pTZAP1. pTZAP1 was restriction analysed to confirm correct orientation of ACP promoter fragment. pTZAP1 was restricted with SacI and Kpn1 and recovered from agarose gel via DEAE paper. The recovered pTZAP1 was ligated with the 764 bp NL2 fragment from above to form pNL4. pNL2 and the plant transformation vector pBI 101 (Clontech) were digested with BamH1 and Sac1. The 2.15kb fragment of pNL2 and the pBI 101 vector fragment

- 21 -

were recovered from DEAE cellulose and ligated to form pETR1. pETR1 was subjected to restriction analysis to confirm correct insertion of gene fragments.

Example 3

Agrobacterium-mediated Transformation of Brassica napus

The vector pETR1 was transferred into Agrobacterium tumefaciens pGV3850 (Zambryski et al., (1983) EMBO J. 2:2143-2150) using a direct DNA uptake procedure [An et al., (1988) In Plant Molecular Biology Manual (Gelvin & Schilperoort, Eds.), Kluwer Academic Publishers, pp. 1-19]. From the resultant Agrobacterium colonies, DNA was extracted, transformed into E. coli from which it was re-isolated to confirm correct gene insertion.

Brassica napus (cv. Westar) stem segments were transformed with the above binary Agrobacterium strain using the procedure of Fry et al. [(1987) Plant Cell Reports 6: 321-325] with the following modifications: kanamycin selection was at 20ug/ml and was delayed until 2 weeks after infection; carbenicillin was replaced by cetotaxime (500ug/ml); arginine was omitted from the regeneration media; 0.8% agar was replaced by 1% agarose; a 2-3 day pre-treatment of stem segments was carried out prior to Agrobacterium infection and a N. plumbaginifolia cell line [Barfield et al. (1985), Plant Cell Reports, 4: 104-107] was used as feeder layer. Shoots formed and remaining green on Plant Cell Reports, 4: 104-107] was used as feeder layer. Shoots formed and remaining green on selective media after 2 transfers were tested for the presence of nopaline [Otten and Schilperoort (1978), Biochem. Biophys. Acta, 527: 497-500]. Positive shoots

- 22 -

were transferred to soil, potted on into 5" diameter pots and transferred to growth rooms operating a 16h day (22°C) and 8h night (18°C) cycle.

Control plants were transformed with A. tumefaciens pGV3850 containing pAPIGUS [de Silva et al. (1992), Plant Molecular Biology, 18: 1163-1172], a plasmid in which the ACP promoter controls the expression of the beta-glucuronidase (GUS) reporter gene.

#### Example 4

##### Analysis of Transformed Plants

DNA was extracted from pETR1 antisense thioesterase plants and from APIGUS transformed control plants and Southern blotted to confirm the presence of inserted genes.

Mature seeds from 39 pETR1 transformed plants and 10 APIGUS transformed plants were analysed for oil content and for fatty acid composition.

After weighing, seeds were macerated in methanol, using an Ultraturrax homogeniser, and extracted overnight in chloroform/methanol (2:1 v/v). After removing seeds by filtration, samples were partitioned with 1% sodium chloride solution. The chloroform-soluble lipid fraction was removed and evaporated to dryness. To analyse for fatty acid composition, extracted lipids from above were refluxed for 60 min with acid methanolysis reagent (toluene/methanol/conc. sulphuric acid 10:20:1). To determine the total fatty acid content of the samples heptadecanoic acid (C17:0) was added as an internal standard (to about 20% of total lipid weight). After

- 23 -

addition of water, the resultant fatty acid methyl esters were extracted with hexane and analysed by gas chromatography on a Carlo Erba chromatograph equipped with a Supelcowax 10/fused silica column.

Table 1 shows the percentage total fatty acid (% TFA) content and the fatty acid composition of seeds from the transgenic plants. The fat content of the control APIGUS seeds (shown as AP1-TAK in Table 1) is seen to range from 38.1%-27.3%. The fat content of the pETR1 antisense thioesterase seeds ranged from 43.2%-14.7%. The reduced oil content of the pETR1 seeds could result from reduced levels of thioesterase activity. Acyl-ACP thioesterase serves as the chain terminating enzyme of de novo fatty acid biosynthesis, cleaving the synthesized acyl-ACP to free fatty acid and ACP. As such, decreasing the level of this terminating enzyme, via antisense technology, could have limited the overall rate of oil synthesis in the seed.

In respect of fatty acid composition, 2 pETR1 plants, 19 and 9, have altered fatty acid compositions of potential commercial use. Seeds from these plants both contain dramatically reduced levels of linolenic acid (18:3) and also significantly reduced levels of linoleic acid (18:2). The production of rape oils containing reduced polyunsaturated fatty acids, such as those from plants 19 and 9, is highly desirable for use as cooking oils since they are less susceptible to oxidation.

#### Example 5

This example describes the construction of plant transformation vectors containing sense rape thioesterase

- 24 -

genes linked to the seed specific ACP promoter for use in altering fatty acid yield and composition in transgenic oil seeds.

To modulate fatty acid biosynthesis within cells of seed tissue by "overexpressing" rape thioesterase, it is necessary to target the thioesterase enzyme to the intracellular site of fatty acid synthesis, namely the plastid. Prior to obtaining a full length thioesterase cDNA, which contained a plastid targetting sequence, a chimaeric thioesterase gene was constructed in which a rape ACP plastid transit sequence was fused to a partial thioesterase cDNA which encoded for the mature enzyme. The chimaeric thioesterase gene was linked to the seed specific rape ACP promoter.

A 361 bp fragment of pNL2, encoding the N-terminal region of the rape thioesterase, was produced from pNL2 by PCR using the primers PTB 26

(5' CTTCTAGATCTCAATGTGCATT, Seq. ID No 19) and PTB 33

(5' CCGAATTCTGCAGTCTCGTCTTCTC, Seq. ID No 20).

PCR reaction (100ul) contained 10ul 10x reaction buffer (Stratagene), 200uM dNTPs, 50 pmole PTB 26, 50 pmole PTB 33, 100ng pNL2, 2.5 units Taq (Stratagene). Mixture heated 92°C for 30 sec, 55°C for 30 sec, 72°C for 60 sec for 25 cycles, followed by 72°C for 10 min for final extension. The PTB 26 primer introduced a XbaI restriction site into the fragment and the PTB 33 primer introduced EcoRI and PstI sites. Following isolation from agarose gels and DEAE paper the PCR fragment was restricted with EcoRI and XbaI, ligated into M13 and DNA sequenced to ensure correct sequence. An EcoRI - BglII version of the PCR fragment was ligated into pNL6 to yield pNL11. pNL6 was constructed by restricting pNL2 with

- 25 -

EcoRI and BglII (partial) recovering the 1.2kb fragment, via DEAE paper; and ligating into EcoRI/BamH1 restricted pTZ19.

A 166 bp ACP transit sequence was obtained from rape ACP cDNA clone 29C08 [Safford et al. (1988), Eur. J. Biochem. 174: 287-295] by PCR amplification using the primers PTB 31

(5' AATGTCGACCACTTCTGCTC, Seq. ID No 21) and PTB 32 (5' TCGAGCTCTGCAGCGCAGGAGAC, Seq. ID No 22).

PTB 31 introduced a SalI site into the fragment and PTB 32 introduced SacI and PstI sites. The PCR product was recovered from agarose gel, via DEAE paper, restricted with SalI and SacI and cloned into M13 to confirm correct DNA sequence. The SalI-SacI fragment was ligated into SalI/SacI restricted pBluescript SK<sup>+</sup> (Stratagene) to form pNL12.

To link the ACP transit sequence to the mature thioesterase sequence pNL11 was restricted with PstI and a 1.1kb fragment recovered and ligated into PstI restricted, phosphatased pNL12. This produced pNL13.

The 1.4kb ACP promoter (API) was released from pTZ5BS by restricting with BamH1 and SalI and was ligated into BamH1/SalI restricted pUCBM20 (Boehringer-Mannheim) to yield pNL14.

To link the ACP promoter to the chimaeric ACP-mature thioesterase sequence pNL13 and pNL14 were both restricted with SalI. A 1.2kb fragment was recovered from pNL13 and ligated into the linearised, phosphatased pNL14 vector to yield pNL15. Following restriction analysis to confirm the correct gene orientation, pNL15 was restricted with

- 26 -

BamH1 and SacI to release a 2.8kb fragment containing the ACP promoter, the ACP transit sequence and the mature thioesterase sequence. This fragment was ligated into the plant transformation vector pBI101 which had been restricted with BamH1 and SacI to remove the b-glucuronidase (GUS) fragment. This final plasmid, pARTE1, was propagated in E.coli and transferred into *A.tumefaciens* pGV3850 via the previously described direct DNA uptake method.

The resulting *A. tumefaciens* strain was used to transform *B. napus* stem segments as described in Example 3 above. From the resulting transgenic plants, seeds will be analysed for altered thioesterase levels and for altered oil content and fatty acid composition.

#### Example 6

Upon obtaining a full length rape acyl-ACP thioesterase cDNA, a plant transformation vector was constructed containing this cDNA linked to the ACP promoter-sequence.

Following EcoRI and partial XbaI restriction of pNL2 1.2kb fragment was recovered onto DEAE paper and ligated into pBluescript SK<sup>+</sup> to yield pNL16. A 180bp fragment containing 5' non-coding thioesterase sequence plus 3 amino acids of the transit peptide was obtained by PCR amplification of pNL5, a rape thioesterase genomic clone, using primers PTB 34

(5' TTG TCG ACA CAC ATG CGT CAT, Seq. ID No 23) and PTB 35

(5' GCA AGC TTC AAC ATT TTT GAT, Seq. ID No 24).

Primer PTB 34 added an additional 6 bp of thioesterase sequence onto the RACE 6 product previously described and

- 27 -

also a SalI restriction site. PTB 35 introduced a HindIII site into the fragment. The 180bp PCR fragment was recovered from DEAE paper restricted with SalI and HindIII and ligated into SalI/HindIII restricted pBluescript SK<sup>+</sup> to give pNL17. DNA sequence analysis was carried out to confirm correct sequence.

A 440bp fragment containing the transit peptide plus part of the mature thioesterase coding sequence was obtained by PCR amplification of pNL2 using primers PTB 36 (5' ACA AGC TTT CGT GTA ATG TGA, Seq. ID No 25) and PTB 37

(5' GTG AAT TCA GAT CTC AAT GTG CAT T, Seq. ID No 26). The PTB 36 primer introduced a HindIII site 3' to the thioesterase initiating ATG and PTB 37 introduced an EcoRI site 3' to the Bgl II site. The 440bp PCR fragment was recovered via DEAE paper, restricted with HindIII and EcoRI and ligated into HindIII/EcoRI restricted M13 to yield pM13HENL2. Fragment was sequenced to confirm correct sequence.

pNL17 was restricted with HindIII/EcoRI and ligated to the 440bp HindIII/EcoRI fragment from pM13HENL2 to yield pNL18. A 560bp SalI-BgIII fragment was recovered from pNL18 and ligated into pNL16 to form pNL19. pNL19 represents a 'full length' thioesterase cDNA.

To link the thioesterase cDNA to the ACP promoter a 1.4kb SalI-SacI fragment was released from pNL19 and ligated into SalI-SacI restricted pNL14 to yield pNL20. pNL20 was restricted with BamH1 and SacI to yield a 2.8kb fragment containing the ACP promoter linked to the thioesterase cDNA. This 2.8kb fragment was ligated into the plant transformation vector pBI101, in which the BamH1-SacI

- 29 -

fragment encoding the  $\beta$ -glucuronidase (GUS) gene had been removed. The final vector, pRATE, was propagated in *E.coli* and transferred into *A. tumefaciens* pGV3850 as previously described.

The resulting *A. tumefaciens* strain was used to transform *B. napus* stem segments as described in Example 3. From the resulting transgenic plants, seeds will be analysed for altered thioesterase levels and for altered oil content and fatty acid composition.

- 29 -

Table I

PLANT NO.	XTPA	C14:0	C16:0	C16:1	C16:2	C16:3	C20:0	C20:1	C22:0	C24:0	C24:1	TRACE	0.5
													0.4
21	43.2	0.0	4.4	2.9	2.3	61.0	58.0	20.6	11.5	10.2	11.2	0.1	0.3
	12	39.9	0.1	4.2	0.3	60.5	19.4	10.2	10.8	11.0	10.0	0.1	0.5
13	38.5	0.0	4.2	0.2	2.1	61.4	18.8	21.8	11.2	12.0	10.5	0.1	0.4
	3	37.7	0.1	4.1	0.3	59.7	20.6	21.8	11.0	11.0	11.0	0.1	0.4
1	37.5	0.1	4.5	0.3	1.6	57.0	21.8	20.9	11.3	11.5	11.4	0.1	0.4
	4	37.4	0.1	4.6	1.6	58.7	21.6	19.1	11.1	11.1	11.0	0.1	0.4
15	36.1	1.5	3.9	2.2	2.2	58.1	21.6	19.1	11.1	11.1	11.0	0.1	0.4
	11	34.7	0.0	4.4	0.3	1.6	59.3	17.3	13.1	9.3	9.3	9.3	0.1
20	32.8	0.1	4.8	0.2	1.8	63.5	21.7	19.1	11.1	11.1	11.0	0.1	0.4
	9	32.7	0.1	4.6	2.4	54.7	21.7	19.1	11.1	11.1	11.0	0.1	0.4
45	31.2	0.1	4.5	1.6	1.6	61.6	19.1	10.0	10.1	10.1	10.0	0.1	0.4
	25	28.4	0.0	4.8	1.5	58.4	21.6	19.1	11.1	11.1	11.0	0.1	0.4
8	27.5	0.0	4.2	2.2	2.2	61.8	18.9	10.7	10.5	10.5	10.5	0.1	0.4
	16	26.8	0.0	4.4	0.3	63.5	17.3	10.5	10.5	10.5	10.5	0.1	0.4
40	26.6	0.0	4.6	0.4	1.3	59.6	20.5	18.2	11.1	11.1	11.0	0.1	0.4
	14	26.4	0.0	4.5	1.4	58.3	21.6	20.6	10.4	10.4	10.4	0.1	0.4
35	26.1	0.0	5.0	0.4	1.4	59.8	21.8	21.8	12.4	12.4	12.4	0.1	0.4
	7b	26.0	0.0	5.0	1.4	57.5	21.8	21.8	11.1	11.1	11.0	0.1	0.4
38	25.9	0.1	4.5	0.3	1.8	56.0	21.8	21.8	12.4	12.4	12.4	0.1	0.4
	18	25.3	0.1	4.5	1.8	56.0	21.8	21.8	11.1	11.1	11.0	0.1	0.4
3b	24.5	0.0	4.9	0.2	1.8	64.0	19.0	16.8	8.4	8.4	8.4	0.1	0.4
	15b	24.2	0.1	4.6	0.2	2.1	61.5	18.8	18.8	8.5	8.5	8.5	0.1
41	24.0	0.1	4.6	0.3	1.9	61.7	19.5	19.5	8.5	8.5	8.5	0.1	0.4
	6	23.3	0.2	6.2	0.4	3.0	56.5	20.0	20.0	10.2	10.2	10.2	0.1
23	23.2	0.1	5.2	0.4	1.9	62.0	19.3	19.3	7.4	7.4	7.4	0.1	0.4
	18b	22.8	0.1	5.0	1.3	56.3	21.9	11.9	9.1	9.1	9.1	0.1	0.4
14b	22.5	0.0	5.2	0.4	2.2	60.6	18.8	18.8	10.0	10.0	10.0	0.1	0.4
	26	21.9	0.0	5.0	1.0	61.4	18.5	18.5	8.0	8.0	8.0	0.1	0.4
7	21.6	0.1	6.1	1.0	1.9	62.8	17.7	17.7	4.3	4.3	4.3	0.1	0.4
	28	20.5	0.0	4.9	0.7	2.1	62.5	66.3	17.9	6.4	6.4	6.4	0.1
9b	20.4	0.0	6.2	0.7	0.7	2.1	63.0	25.1	9.6	9.6	9.6	0.1	0.4
	36	18.0	0.1	6.1	0.8	1.9	69.0	12.3	3.8	1.1	1.1	1.1	0.1
46	16.5	0.1	5.8	0.4	2.6	69.0	22.1	8.0	0.8	0.8	0.8	0.1	0.4
	19	15.7	0.1	5.8	0.9	2.0	55.5	22.1	22.1	22.1	22.1	22.1	0.1
24	14.7	0.2	6.7	0.9	0.9	55.5	22.1	22.1	22.1	22.1	22.1	0.1	0.4
	2	14.7	0.2	6.7	0.9	0.9	55.5	22.1	22.1	22.1	22.1	22.1	0.1
10	38.1	0.0	4.6	0.3	2.2	61.6	17.9	17.9	9.7	9.7	9.7	0.1	0.5
	4	35.7	0.1	4.4	0.3	2.0	61.3	18.5	10.2	10.2	10.2	10.2	0.1
3	34.3	0.1	4.3	0.3	1.6	59.0	19.6	11.6	11.6	11.6	11.6	0.1	0.5
	6	33.4	0.0	4.6	0.3	2.2	62.8	17.9	10.4	10.4	10.4	10.4	0.1
1	33.7	0.0	4.5	0.4	2.0	59.1	17.9	11.6	11.6	11.6	11.6	0.1	0.5
	1	32.9	0.1	4.4	0.3	1.8	53.7	23.3	12.8	12.8	12.8	12.8	0.1
2	32.1	0.0	4.4	0.4	2.0	69.8	24.7	13.7	13.7	13.7	13.7	0.1	0.5
	7	31.0	0.1	4.6	0.3	1.8	53.7	23.3	12.8	12.8	12.8	12.8	0.1
9	29.3	0.1	5.3	0.6	2.0	69.8	24.7	13.7	13.7	13.7	13.7	0.1	0.5
	11	29.3	0.1	5.4	0.6	2.0	48.1	26.7	26.7	26.7	26.7	26.7	0.1
8	27.3	0.1	5.4	0.6	2.0	55.5	26.7	26.7	26.7	26.7	26.7	0.1	0.5
	6	27.3	0.1	5.4	0.6	2.0	55.5	26.7	26.7	26.7	26.7	26.7	0.1
AP1-TAK	58.2	20.2	20.2	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5	11.5
	2.3	2.4	2.4	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
XTPA	58.1	0.0	4.6	0.3	2.0	61.6	17.9	17.9	9.7	9.7	9.7	9.7	9.7
	4	3	3	3	2.0	61.3	18.5	18.5	10.2	10.2	10.2	10.2	10.2

- 30 -

CLAIMS

1. A nucleotide sequence encoding an enzyme precursor having acyl-ACP thioesterase activity, comprising nucleotides 169-1269 of the sequence shown in Figure 1 (Seq. ID No 1) or functional equivalents thereof.
2. A sequence according to claim 1, comprising nucleotides 169-1269 of the sequence shown in Figure 2 (Seq. ID No 3).
3. A sequence according to claim 1 or 2, further comprising a 5' untranslated region.
4. A sequence according to claim 1, 2 or 3, further comprising a 3' untranslated region.
5. A sequence according to any one of the preceding claims, comprising the sequence obtainable from Brassica napus.
6. A sequence according to any one of the preceding claims, further comprising a seed-specific promoter.
7. A polypeptide having acyl-ACP thioesterase activity, comprising the amino acid sequence shown in Figure 1 (Seq. ID No 1) or functional equivalents thereof.
8. A polypeptide according to claim 7, comprising the amino acid sequence shown in Figure 2 (Seq. ID No 3).
9. A vector comprising a nucleotide sequence according to any one of claims 1-6.

- 31 -

10. A host cell into which has been introduced the sequence of any one of claims 1-6.

11. A plant host cell according to claim 10, which comprises part of, or is capable of giving rise to, a plant.

12. A method of altering the characteristics of a plant, comprising introducing into the plant the sequence of any one of claims 1-6, so as to alter the level of acyl-ACP thioesterase activity.

13. A method according to claim 12, wherein the characteristics so altered comprise the fatty acid yield and/or fatty acid composition of the plant.

14. A method according to claim 12 or 13, wherein the yield and/or composition of seed storage oil of the plant is altered.

15. A method of producing an enzyme having acyl-ACP thioesterase activity, comprising: inserting the sequence of any one of claims 1-6 capable of expressing the enzyme into a suitable expression vector; transforming a host cell with said vector; growing said transformed host cell in suitable culture conditions; and obtaining the enzyme from the host cells and/or the culture medium.

16. A method according to claim 15, wherein the host cell is a microorganism.

1/12

## RAPE ACYL-ACP THIOESTERASE CLONE PNL2

v10            v20            v30            v40  
 GGC GTC ATTGCCAATTGGCGAAACAACCAAGCTTTTAATGGCAATAT  
 v50            v60            v70            v80            v90            v100  
 CGT ATTTACTAACCTCCACCTCCTCGTTAACATCGAGCTTCAGATAACACAC  
 v110            v120            v130            v140            v150  
 CAGATCCCTGATTCTCTTCTATAAAACCAACCTCAGGAACCATAAAAAAAA  
 v160            v170            v180            v190            v200  
 GGGCATCAAAA ATG TTG AAG CTT TCG TGT AAT GTG ACT AAC CAC  
 MET LEU LYS LEU SER CYS ASN VAL THR ASN HIS  
 v210            v220            v230            v240  
 TTA CAC ACC TTC TCC TTC TCC GAT TCC TCC CTT TTC ATC  
 LEU HIS THR PHE SER PHE SER ASP SER SER LEU PHE ILE  
 v250            v260            v270            v280  
 CCG GTT AAT CGC CGT ACC CTC GCC GTC TCG TCT TCT CAG CCA  
 PRO VAL ASN ARG ARG THR LEU ALA VAL SER SER GLN PRO  
 v290            v300            v310            v320  
 AGG AAG CCG GCT TTA GAT CCT CTT CGG GCA GTT ATC TCC GCC  
 ARG LYS PRO ALA LEU ASP PRO LEU ARG ALA VAL ILE SER ALA  
 v330            v340            v350            v360  
 GAT CAG GGA AGC ATC AGC CCT GTT AAT TCG TGT ACC CCG GCG  
 ASP GLN GLY SER ILE SER PRO VAL ASN SER CYS THR PRO ALA  
 v370            v380            v390            v400            v410  
 GAT CGG TTC CGA GCT GGT CGA TTG ATG GAA GAT GGT TAT TCT  
 ASP ARG PHE ARG ALA GLY ARG LEU MET GLU ASP GLY TYR SER  
 v420            v430            v440            v450  
 TAC AAA GAG AAG TTC ATT GTT AGA AGC TAT GAG GTT GGG ATT  
 TYR LYS GLU LYS PHE ILE VAL ARG SER TYR GLU VAL GLY ILE  
 v460            v470            v480            v490  
 AAC AAA ACC GCC ACC GTC GAG ACA ATT GCT AAT CTC TTA CAG  
 ASN LYS THR ALA THR VAL GLU THR ILE ALA ASN LEU LEU GLN  
 v500            v510            v520            v530  
 GAG GTG GCA TGT AAC CAT GTT CAG AAG TGT GGA TTC TCG ACC  
 GLU VAL ALA CYS ASN HIS VAL GLN LYS CYS GLY PHE SER THR  
 v540            v550            v560            v570  
 GAT GGA TTT GCC ACA ACA CTC ACC ATG AGG AAA TTG CAT CTC  
 ASP GLY PHE ALA THR THR LEU THR MET ARG LYS LEU HIS LEU  
 v580            v590            v600            v610            v620  
 ATA TGG GTC ACT GCA AGA ATG CAC ATT GAG ATC TAC AAG TAC  
 ILE TRP VAL THR ALA ARG MET HIS ILE GLU ILE TYR LYS TYR

2/12

V630	V640	V650	V660	
CCA GCT TGG AGT GAT GTT GAG ATA GAG ACA TGG TGC CAG				
PRO ALA TRP SER ASP VAL VAL GLU ILE GLU THR TRP CYS GLN				
V670	V680	V690	V700	
AGT GAA GGA AGG ATT GGA ACG AGA CGT GAT TGG ATT CTA AGG				
SER GLU GLY ARG ILE GLY THR ARG ARG ASP TRP ILE LEU ARG				
V710	V720	V730	V740	
GAC TCT GCT ACA AAT GAA GTT ATT GGG CGT GCT ACA AGC AAG				
ASP SER ALA THR ASN GLU VAL ILE GLY ARG ALA THR SER LYS				
V750	V760	V770	V780	
TGG GTG ATG ATG AAC CAA GAC ACA AGG CGG CTT CAA AGA GTT				
TRP VAL MET MET ASN GLN ASP THR ARG ARG LEU GLN ARG VAL				
V790	V800	V810	V820	V830
ACA GAT GAA GTT CGG GAC GAG TAC TTG GTT TTC TGT CCT CGA				
THR ASP GLU VAL ARG ASP GLU TYR LEU VAL PHE CYS PRO ARG				
V840	V850	V860	V870	
GAA CCC AGA CTA GCG TTT CCA GAA GAG AAC AAT AGC AGC TTA				
GLU PRO ARG LEU ALA PHE PRO GLU GLU ASN ASN SER SER LEU				
V880	V890	V900	V910	
AAG AAA ATC CCA AAA CTA GAA GAT CCA GCT CAG TAT TCT ATG				
LYS LYS ILE PRO LYS LEU GLU ASP PRO ALA GLN TYR SER MET				
V920	V930	V940	V950	
CTA GAG CTT AAG CCT CGG CGA GCT GAT CTG GAC ATG AAC CAG				
LEU GLU LEU LYS PRO ARG ARG ALA ASP LEU ASP MET ASN GLN				
V960	V970	V980	V990	
CAC GTG AAT AAC GTC ACC TAC ATT GGA TGG GTG CTT GAG AGC				
HIS VAL ASN ASN VAL THR TYR ILE GLY TRP VAL LEU GLU SER				
V1000	V1010	V1020	V1030	V1040
ATA CCT CAA GAA ATC ATT GAT ACG CAT GAG CTT CAA GTT ATA				
ILE PRO GLN GLU ILE ILE ASP THR HIS GLU LEU GLN VAL ILE				
V1050	V1060	V1070	V1080	
ACT CTA GAT TAC AGA AGA GAA TGC CAG CAA GAT GAC ATT GTA				
THR LEU ASP TYR ARG ARG GLU CYS GLN GLN ASP ASP ILE VAL				
V1090	V1100	V1110	V1120	
GAT TCA CTC ACC ACC TCT GAA ATC CCT GAC GAC CCG ATC TCA				
ASP SER LEU THR SER GLU ILE PRO ASP ASP PRO ILE SER				
V1130	V1140	V1150	V1160	
AAG CTT ACC GGG ACC AAC GGA TCT GCC ACG TCA AGC ATA CAA				
LYS LEU THR GLY THR ASN GLY SER ALA THR SER SER ILE GLN				
V1170	V1180	V1190	V1200	
GGA CAC AAT GAG AGC CAA TTC TTG CAT ATG CTG AGG TTG TCA				
GLY HIS ASN GLU SER GLN PHE LEU HIS MET LEU ARG LEU SER				

3/12

v1210        v1220        v1230        v1240        v1250  
 GAA AAT GGC CAG GAG ATC AAT CGT GGA AGA ACA CAA TGG AGA  
 GLU ASN GLY GLN GLU ILE ASN ARG GLY ARG THR GLN TRP ARG  
  
 v1260        v1270        v1280        v1290        v1300  
 AAG AAA TCC TCA CGA TGATTTCTTATATGCTTCTCCAGTGTGGTGAAG  
 LYS LYS SER SER ARG TER  
  
 v1310        v1320        v1330        v1340        v1350  
 TTCTTGCTTCACGTTATAGTTTATTGTGTCTTTGATCAGATCTGTTGTGGGG  
  
 v1360        v1370        v1380        v1390        v1400        v1410  
 TTAGATGGTACTGGATTATTTGTGTAGTGTGGCGTCTGTGTTGTTCTGT  
  
 v1420        v1430        v1440        v1450        v1460  
 CCAAATTTGGGCTTAAGCAAAACGTTCTTAGGTCTGCTTGTAGCTTTCTGT  
  
 v1470        v1480        v1490        v1500        v1510        v1520  
 AACTAGATTGTTCATCCAATTATATGCTGAGTTCTGTGATGAAACTGGGTA  
  
 v1530        v1540        v1550        v1560        v1570  
 TGTGATGTTGGAGGCAGAAATTGACACAATTAAAATTGTATTACATAAGTTTTT  
  
 v1580        v1590        v1600        v1610        v1620        v1630  
 GGATAAGCTAAAGTTGTATGCAATGTAATACTTTTTATTGGAGATTAATA  
  
 v1640        v1650        v1660        v1670        v1680  
 ATATTTAAATAAAAAAAAAAAAAAA  
  
 v1690        v1700        v1710  
 AAAAAAAAAAAAAACTCGAG

Fig.1 Sheet 3

4/12

## RAPE ACYL-ACP THIOESTERASE CLONE PNL3

v10            v20            v30            v40  
 GGC GTC ATTGCCAATTGGCGAACACACCAGCTTTAATGGCAATAT  
 v50            v60            v70            v80            v90            v100  
 CGT ATTTTACTAATCTCCACCTCCTCGTTAACATCGAGCTTCAGATAACAACACA  
 v110            v120            v130            v140            v150  
 CAG ATCCCTGATTCTCTTCTATAAAACCAAAACCTCAGGAACCATAAAAAAAA  
 v160            v170            v180            v190            v200  
 GGG CATCAAAA ATG TTG AAG CTT TCG TGT AAT GTG ACT AAC AAC  
 MET LEU LYS LEU SER CYS ASN VAL THR ASN ASN  
 v210            v220            v230            v240  
 TTA CAC ACC TTC TCC TTC GAT TCC TCC CTT TTC ATC  
 LEU HIS THR PHE SER PHE SER ASP SER SER LEU PHE ILE  
 v250            v260            v270            v280  
 CCG GTT AAT CGC CGT ACC ATC GCC GTC TCG TCT TCT CAG CTA  
 PRO VAL ASN ARG ARG THR ILE ALA VAL SER SER GLN LEU  
 v290            v300            v310            v320  
 AGG AAG CCG GCT TTA GAT CCT CTA CGG GCA GTT ATC TCC GCG  
 ARG LYS PRO ALA LEU ASP PRO LEU ARG ALA VAL ILE SER ALA  
 v330            v340            v350            v360  
 GAT CAG GGA AGC ATC AGC CCT GTT AAT TCG TGT ACA CCG GCG  
 ASP GLN GLY SER ILE SER PRO VAL ASN SER CYS THR PRO ALA  
 v370            v380            v390            v400            v410  
 GAT CGG TTA CGA GCT GGT CGA TTG ATG GAA GAT GGT TAT TCG  
 ASP ARG LEU ARG ALA GLY ARG LEU MET GLU ASP GLY TYR SER  
 v420            v430            v440            v450  
 TAC AAA GAG AAG TTC ATT GTT AGA AGC TAT GAG GTT GGG ATT  
 TYR LYS GLU LYS PHE ILE VAL ARG SER TYR GLU VAL GLY ILE  
 v460            v470            v480            v490  
 AAC AAA ACC GCC ACC GTC GAG ACA ATT GCT AAT CTC TTA CAG  
 ASN LYS THR ALA THR VAL GLU THR ILE ALA ASN LEU LEU GLN  
 v500            v510            v520            v530  
 GAG GTG GCG TGT AAC CAT GTT CAG AAG TGT GGA TTC TCG ACG  
 GLU VAL ALA CYS ASN HIS VAL GLN LYS CYS GLY PHE SER THR  
 v540            v550            v560            v570  
 GAT GGA TTT GCC ACA ACA CTC ACC ATG AGG AAA TTG CAT CTC  
 ASP GLY PHE ALA THR THR LEU THR MET ARG Lys LEU HIS LEU  
 v580            v590            v600            v610            v620  
 ATA TGG GTC ACT GCA AGA ATG CAC ATT GAG ATC TAC AAA TAT  
 ILE TRP VAL THR ALA ARG MET HIS ILE GLU ILE TYR LYS TYR

5/12

CCA GCT TGG AGT GAT GTT GAG ATA GAG ACA TGG TGC CAG	v630	v640	v650	v660
Pro Ala Trp Ser Asp Val Val Glu Ile Glu Thr Trp Cys Gln				
ACT GAA GGA AGG ATT GGA ACG AGA CGT GAT TGG ATT CTA AGG	v670	v680	v690	v700
Ser Glu Gly Arg Ile Gly Thr Arg Arg Asp Trp Ile Leu Arg				
GAC TCT GCT ACA AAT GAA GTT ATT GGA CGT GCT ACA AGC AAG	v710	v720	v730	v740
Asp Ser Ala Thr Asn Glu Val Ile Gly Arg Ala Thr Ser Lys				
TGG GTG ATG ATG AAC CAA GAC ACA AGG CGG CTT CAA AGA GTT	v750	v760	v770	v780
Trp Val Met Met Asn Gln Asp Thr Arg Arg Leu Gln Arg Val				
ACA GAT GAA GTT CGG GAC GAG TAC TTG GTT TTC TGT CCT CGA	v790	v800	v810	v820
Thr Asp Glu Val Arg Asp Glu Tyr Leu Val Phe Cys Pro Arg				
GAA CCC AGA CTA CGG TTT CCA GAA GAG AAC AAT AGC AGC TTA	v840	v850	v860	v870
Glu Pro Arg Leu Ala Phe Pro Glu Asn Asn Ser Ser Leu				
AAG AAA ATC CCA AAA CTA GAA GAT CCA GCT CAG TAT TCT ATG	v880	v890	v900	v910
Lys Lys Ile Pro Lys Leu Glu Asp Pro Ala Gln Tyr Ser Met				

Fig. 2,  
Sheet 2

6/12

TA GAG CTT AAG CCT CGG CGA GCT GAT CTG GAC ATG AAC CAG	v920	v930	v940	v950
eu Glu Leu Lys Pro Arg Arg Ala Asp Leu Asp Met Asn Gln				
AC GTG AAT AAC GTC ACC TAC ATC GGA TGG GTG CTT GAG AGC	v960	v970	v980	v990
is Val Asn Asn Val Thr Tyr Ile Gly Trp Val Leu Glu Ser				
TA CCT CAA GAA ATC ATT GAT ACG CAT GAG CTT CAA GTT ATA	1000	v1010	v1020	v1030
le Pro Gln Glu Ile Asp Ile Asp Thr His Glu Leu Gln Val Ile				
CT CTA GAT TAC AGA AGA GAA TGC CAG CAA GAT GAC ATT GTA	v1050	v1060	v1070	v1080
hr Leu Asp Tyr Arg Arg Glu Cys Gln Gln Asp Asp Ile Val				
AT TCA CTC ACC ACC TCT GAA ATC CCT GAC GAC CCG ATC TCA	v1090	v1100	v1110	v1120
sp Ser Leu Thr Thr Ser Glu Ile Pro Asp Asp Pro Ile Ser				
AG TTT ACC GGG ACC AAC GGA TCT GCC ATG TCA AGC ATA CAA	v1130	v1140	v1150	v1160
ys Phe Thr Gly Thr Asn Gly Ser Ala Met Ser Ser Ile Gln				
GA CAC AAT GAG AGC CAG TTC TTG CAT ATG CTG AGG TTG TCA	v1170	v1180	v1190	v1200
ly His Asn Glu Ser Gln Phe Leu His Met Leu Arg Leu Ser				

Fig. 2, Sheet 3

7/12

v1210                    v1220                    v1230                    v1240                    v1250  
 GAA AAT GGC CAG GAG ATC AAT CGT GGG AGA ACA CAA TGG AGA  
 GLU ASN GLY GLN GLU ILE ASN ARG GLY ARG THR GLN TRP ARG  
  
 v1260                    v1270                    v1280                    v1290                    v1300  
 AAG AAA TCC TCA CGA TGATTCTTATGCTTCAGTGGTGAAG  
 LYS LYS SER SER ARG TER  
  
 v1310                    v1320                    v1330                    v1340                    v1350  
 TTCTTGCTTACGTTTATAGTTTATTGTCTCTTTGATCAGATCTATTGTGGGG  
  
 v1360                    v1370                    v1380                    v1390                    v1400                    v1410  
 TTTAGATGGTACTGGATTATTGTAGTAGTGTGGCGTCTGTGTTATTTCGTTTCG  
  
 v1420                    v1430                    v1440                    v1450                    v1460  
 TCCAAATTGTTGGGCTTTAAGCAAACGTTCTTAGGTCTTAATCTTTGATCCGC  
  
 v1470                    v1480                    v1490                    v1500                    v1510                    v1520  
 TTGTATCTTTCATAAACTAGATTTCATCCATTATATGCTGAGTTTCCTC  
  
 v1530                    v1540                    v1550                    v1560                    v1570  
 TTAAAAAAAGAAAAAAAGAAAAAAAGAAAAAAAGAAAAAAAGAAAAAAAGAAAAAA  
 v1580                    AAAACTCGAG

Fig. 2, Sheet 4

RAPE ACYL-ACP THIOESTERASE

GGCGTCAATTGCCAATTGGGAAACCAACCAGCTTTAATGGCAATAATCGTATTACTAATCTCCACCTTCCTCGTTAAC	80	
	80	
ATCGAGGCTTCAGATAACACAGATCCCTGATTCCTCTTACCAACCTCAGGAACCATAAAAAAGGG	160	
	160	
CATCAAAATGTGAAAGCTTCTCGTAATGTGACTAACCACTTACACACCTTCTCCGATTCCCTTCCTTC	240	
	240	
ATCCCGGTTAACCGCTTCTCGTCTCGCCGATCCGGGCTTAGATCCTCTCCGAGTCGGCAGTTAT	320	
	320	
CTCCGGCGATCAGGAAAGCATCAGGCCCTGTTAACATTCTCGTGTACCCCCGGATCGGTTCGGAGCTGGTCAATGGAAG	400	
	400	
ATGGTTATTCTACAGAGAAGTTCAATTGAGCTATGGGATTAAACAAAACGCCACCGTCGAGACAATT	480	
	480	
GCTAATCTCTTACAGGAGGTGGCATGTAACCATGTTCAAGAAGTGTGGATTCTCGACCCGATGGATTGCCACACACTCAC	560	
	560	
CATGAGGAATTGCATCTCATGGTCACTGGTACAGTACATGAGATCTACAAGTACCGCTGGAGTGATGTG	640	
	640	
TGAGATAGAGACATGGTGGCCAGAGTGAAGGAAGGATTGGATTCAGGACTCTGCTACAAAT	720	
	720	
GAAGTTATTGGGCGTACAAGCAAGTGGGTGATGAAACCAAGACACAGGGCTTCAGAGTTACAGATGAGT	800	
	800	
TGGGACGAGTACTTGGTTCTCGAGAACCCAGACTAGCGTAAAGAGAAACAAATAGCAGCTTAAGAAAA	880	
	880	

**Fig. 3, Sheet 1**

Fig. 3, Sheet 2

10/12

RAPE ACYL-ACP THIOESTERASE

NL2	MLKLSCNVTNHLHTFSFFSDSSLFIPVNRRTLAVSSSQPRKPALDPLRAV	50
NL3	<u>N</u> I-----L-----	50
NL2	ISADQGSISPVNNSCTPADRFRAGRLEMEDGYSYKEKFIVRSYEVGINKTAT	100
NL3	<u>L</u> -----	100
NL2	VETIANLLQEVAACNHVQKCGFSTDGFATTLMRKLHLIWVTARMHIEIYK	150
NL3	-----	150
NL2	YPAWSDVVEIETWCQSEGREGTRRDWILRDSATNEVIGRATSKWVMMNQD	200
NL3	-----	200
NL2	TRRLQRVTDEVRDEYLVFCPREPRLAFPEENNSSLKKIPKLEDPAQYSML	250
NL3	-----	250
NL2	ELKPRRADLDMNQHVNNVTYIGWVLESIPQEIIDTHELQVITLDYRRECQ	300
NL3	-----	300
NL2	QDDIVDSLTTSEIPDDPISKLTGTNGSATSSIQGHNESQFLHMLRLSENG	350
NL3	<u>F</u> M-----	350
NL2	QEINRGRTQWRKKSSR	366
NL3	-----	366

Fig.4

11/12

RAPE THIOESTERASE PEPTIDE SEQUENCESENDOPROTEINASE LYS-C

PEPTIDE 26            F I V R S Y E V G I N K

TRYPSIN

PEPTIDE 15            L S [G D] G Q E I N R

PEPTIDE 30            L A F P E E N N [R]

PEPTIDE 34            V M M N Q D T R

PEPTIDE 39            D W I L R

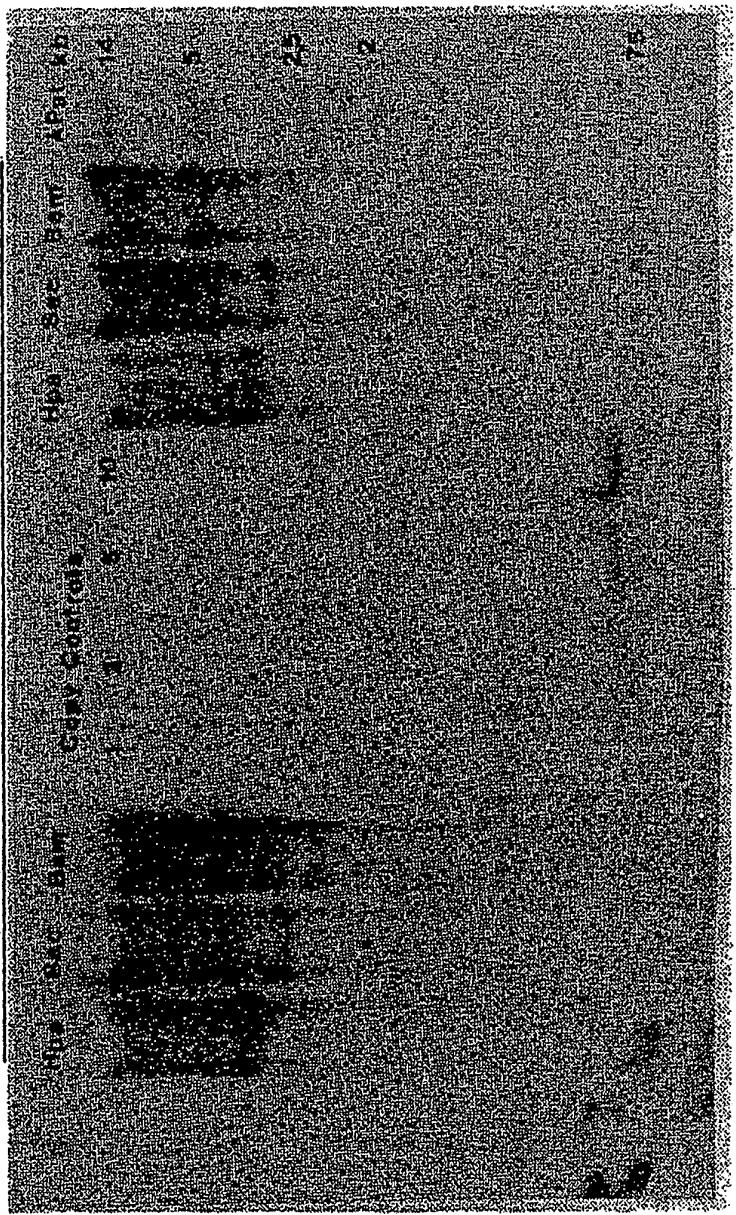
Fig. 5

12/12

**RAPE THIOESTERASE**

---

**GENOMIC SOUTHERN BLOT**



HpaI	-	12 copies per haploid genome
SacI	-	17 copies
BamHI	-	16 copies

**Fig. 6**